ELSEVIER

Biochimica et Biophysica Acta 1270 (1995) 26–35

BBA Biochimica et Biophysica Acta

Irreversibly glycated albumin alters the physico-chemical characteristics of low density lipoproteins of normal and diabetic subjects

Anca Dobrian, Maya Simionescu *

Institute of Cellular Biology and Pathology, 8 B.P. Hasdeu Str., 79691 Bucharest, Romania

Received 13 June 1994; revised 19 September 1994

Abstract

In diabetic plasma, glycated albumin and glycated LDL coexist with augmented levels of peroxides, conditions frequently associated with the development of accelerated atherosclerosis. The direct interaction between irreversibly glycated albumin, LDL and oxidation have not been explored yet. We tried to elucidate whether irreversibly glycated albumin (AGE-Alb) induces changes in the chemistry and morphology of LDL particle, and if AGE-Alb has the ability to scavenge free radicals, as reported for native albumin. LDL isolated from normal (nLDL) or diabetic human subjects (dLDL) was incubated in vitro with AGE-Alb in conditions of autoxidation (37°C, 24–48 h in the absence of oxidation inhibitors) or of Cu^{2+} induced-oxidation. The results showed that, especially in the latter condition, AGE-Alb induced marked physico-chemical modifications of both nLDL and dLDL without significant changes in the level of peroxides. Incubation with AGE-Alb decreased the cholesteryl esters/unesterified cholesterol ratio of nLDL by 30% and of dLDL by ~50%. Concomitantly, in oxidative conditions a marked increase (~3-fold) in the lysophosphatidylcholine/phosphatidylcholine ratio of dLDL was detected. Apolipoprotein B integrity as well as the morphology of the lipoprotein particles were drastically affected. To a lesser extent, these modifications occurred also in the presence of inhibitors of oxidation at 37°C, but not at 4°C. The above described effects were constantly more pronounced in the case of dLDL. These results indicated that in the absence of other plasma or vascular tissue components (e.g., endothelial cells, extracellular matrix) AGE-Alb by itself induces alterations in the chemistry and morphology of LDL, especially of glycated LDL, modifications that may account for the occurrence of accelerated atherogenesis in diabetes.

Keywords: Advanced glycosylation end product; Diabetes; Atherosclerosis; Oxidation; Lipid peroxide; (Human)

1. Introduction

It is well documented that accelerated atherosclerosis is the main complication of diabetes. Non-enzymatic glycation of proteins and increased concentration of plasma peroxides are common events in the pathogenesis of diabetes mellitus and its chronic complications [1–3]. Moreover, it was suggested that increased glycation may predispose proteins to oxidative damage [4], which can further impair the properties and functions of such altered proteins. Accumulation of advanced glycosylation end products (AGEs) is particularly critical for long-lived structural proteins, such as albumin. Irreversibly glycated albumin (AGE-Alb) has been demonstrated to undergo conformational alterations, associated with decreased ligand binding capacity [5], the capability to generate superoxides and a weak scavenging activity against peroxides [6]. Exposure to glucose also affects short-lived proteins, such as plasma low density lipoproteins (LDL) decreasing their interaction (in vitro) with the fibroblast LDL receptors [7]. Recent evidence indicated that the Maillard reaction represents a combination of both glycative and oxidative damage of proteins, yielding glycoxidation products [8]. The entire process might be especially precarious if these lipoproteins become sequestered in the vessel wall. In diabetic patients, the presence of non-enzymatically bound glucose on lipoproteins such as LDL [9], may increase the likelihood of oxidative alterations, which could affect both the apoprotein and the lipid moiety of the particle. Oxidatively modified LDL detected in atherosclerotic plaques in vivo [10] was demonstrated to be highly atherogenic, cytotoxic to the cells of the vessel wall [11], and stimulator of foam cell formation [12].

In normal conditions, a number of antioxidants are present in the plasma, i.e., ascorbic acid, superoxide dis-

* Corresponding author. Fax: +40 1 3121143.

SSDI 0925-4439(94)00068-9

mutase, vitamin E, ceruloplasmin, transferrin, albumin, the latter being one of the most potent. Albumin is able to chelate traces of metal ions and to scavenge free radicals [13,14]. We have recently reported that, during in vitro peroxidation, albumin efficiently prevents the oxidative modifications of LDL and the formation of modified and reassembled lipoproteins [15].

It was reported that in diabetes, the plasma level of free radical scavengers is decreased, while the thiobarbituric acid-reactive substances (TBARS) values are increased [16], and circulating (non-enzymatic reversibly and irreversibly) glycated albumins occur [17]. In addition, the LDL is partly glycated and the Cu^{2+} total levels are higher in diabetics than in normal individuals reaching the highest level in diabetic patients with angiopathy and alterations in lipid metabolism [18]. However, no data are available so far on the possible direct effects of AGE-Alb on LDL (normal or glycated), or whether glycation is of pathophysiological significance in the absence of other plasma and tissue components.

Bearing on the above mentioned data, we asked the questions whether (a) in a cell-free system, devoid of any component of plasma or extracellular matrix, AGE-Alb has a direct effect on LDL exposed to mild (autoxidation) or strong (copper-induced) oxidation; (b) if LDL obtained from normal (nLDL) or diabetic patients (dLDL) with non-insulin dependent diabetes mellitus (NIDDM) respond differently when exposed to AGE-Alb, and (c) if AGE-Alb maintains the capacity (previously ascribed to native albumin) to prevent the in vitro peroxidation of LDL.

We report here that AGE-Alb induces physico-chemical alterations of LDL, which are particularly prominent for dLDL and specially pronounced in oxidative conditions. Like native albumin, but to a lesser extent, AGE-Alb is still able to prevent TBARS formation during LDL peroxidation.

2. Materials and methods

2.1. Reagents

Chemicals were obtained from the following sources: butylated hydroxytoluene (BHT), phenylmethylsulfonylfluoride (PMSF), disodium ethylenediamine tetraacetic acid (EDTA), sucrose, Tris (hydroxymethylaminomethane), β-mercaptoethanol, acrylamide, sodium dodecylsulfate (SDS), sodium phosphotungstate, 2-thiobarbituric acid, Folin-Ciocalteu phenol reagent and cholesterol enzymatic kit from Sigma (St. Louis, MO, USA), trichloroacetic acid and glucose from Fluka (Buchs, Switzerland). All organic solvents and chromatography plates Kieselgel 60 for thin-layer chromatography (TLC) were from Merck (Darmstadt, Germany), bovine serum albumin (Cohn fraction V) was from Serva (Heidelberg, Germany).

2.2. Probes

Isolation of human serum LDL

From fresh human sera of normal and type I diabetic subjects, LDL was isolated by density gradient ultracentrifugation (within a density cut-off of 1.019 to 1.063 g/ml) according to Redgrave et al. [19]. Both nLDL and dLDL were dialyzed against 10 mM Tris-buffered saline (pH 7.4) for 24 h at 4°C in the presence of 0.01% EDTA and 0.22 mM BHT. The two inhibitors were omitted from the buffer solution when LDL probes were subjected to oxidation. The stable but reversible Amadori products present in preparations of nLDL and dLDL were determined by the thiobarbituric acid (TBA) method using as standard hydroxymethyl furfural (HMF) in concentrations ranging from $1.0 \cdot 10^{-7}$ M to $1.0 \cdot 10^{-5}$ M [20].

Preparation of AGE-albumin

To obtain advanced glycosylation endproducts (AGEs), nonenzymatic glycosylation of albumin (Alb) was conducted as indicated in [21,22]. Briefly, albumin (4%) was dissolved in 0.15 M phosphate-buffered saline (PBS) pH 7.4 supplemented with 150 mM glucose in the presence of 1.5 mM PMSF, penicillin (100 units/ml) and gentamycin (40 μg/ml). After passing through a 0.22 μm Millipore filter, the Alb solution was kept in a dry heat-sterilized tube at 37°C for 60 days. In some experiments, the AGE-Alb preparation was passed through a GlycoGel B column to remove reversibly glycated Alb. AGE-Alb was characterised by spectrofluorimetry [23] and by SDS-PAGE for protein purity. For control, Alb was incubated in the same conditions except that glucose was omitted.

2.3. Incubation of normal and diabetic LDL with AGE-Alb

The experimental protocols carried out consisted in incubating either nLDL or dLDL, in the presence or absence of AGE-Alb or of native Alb (as control), in one of the following conditions: (a) autoxidation, keeping the samples at 24–48 h at 37°C [24], (b) copper-induced oxidation, using 1 μM copper sulfate in the same conditions as in (a) [25] and (c) autoxidation in the presence of 1 mM EDTA and 0.22 mM BHT at 4°C or 37°C. For all experiments, 0.1 mg protein LDL/ml was incubated with different albumins in concentrations ranging from 0.01–1.0 mg/ml in 10 mM Tris-buffered saline (pH 7.4). Oxidation was arrested by adding 1 mM EDTA and 0.22 mM BHT to each sample, and placing the tubes at 4°C. To ascertain the effect of the oxidative conditions, in separate experiments the probes were incubated in the presence or absence of AGE-Alb, at 4°C or at 37°C in the presence of 1 mM EDTA and 0.22 mM BHT. For each condition, after the TBARS were assayed, the LDL was isolated again from the incubation mixture by ultracentrifugation (as described above) and examined by negative staining electron microscopy and analysed for lipid and protein composition.

To determine whether the process is dependent on copper concentration, additional experiments were carried out using various copper/AGE-Alb (or Alb) molar ratio (from 0 to 4) while maintaining constant the albumin/protein LDL weight ratio at 1.

2.4. Investigations

Assay for lipid peroxides

The degree of oxidative modification of LDL was determined by measuring the thiobarbituric acid reactive substances (TBARS), using a malondialdehyde (MDA) standard curve; results were expressed as nmol MDA/mg protein LDL, as described in [26]. Alternatively the conjugated dienes were determined spectrophotometrically according to the method described by Esterbauer [27].

Protein analysis

Protein concentration was determined by a modified Lowry method [28] using bovine serum albumin as standard. For gel electrophoresis, samples of each LDL fraction were precipitated with 7% trichloroacetic acid and 0.015% sodium deoxycholate (final concentration), and heated at 100°C (for 3 min) in 0.06 M Tris-buffered (pH

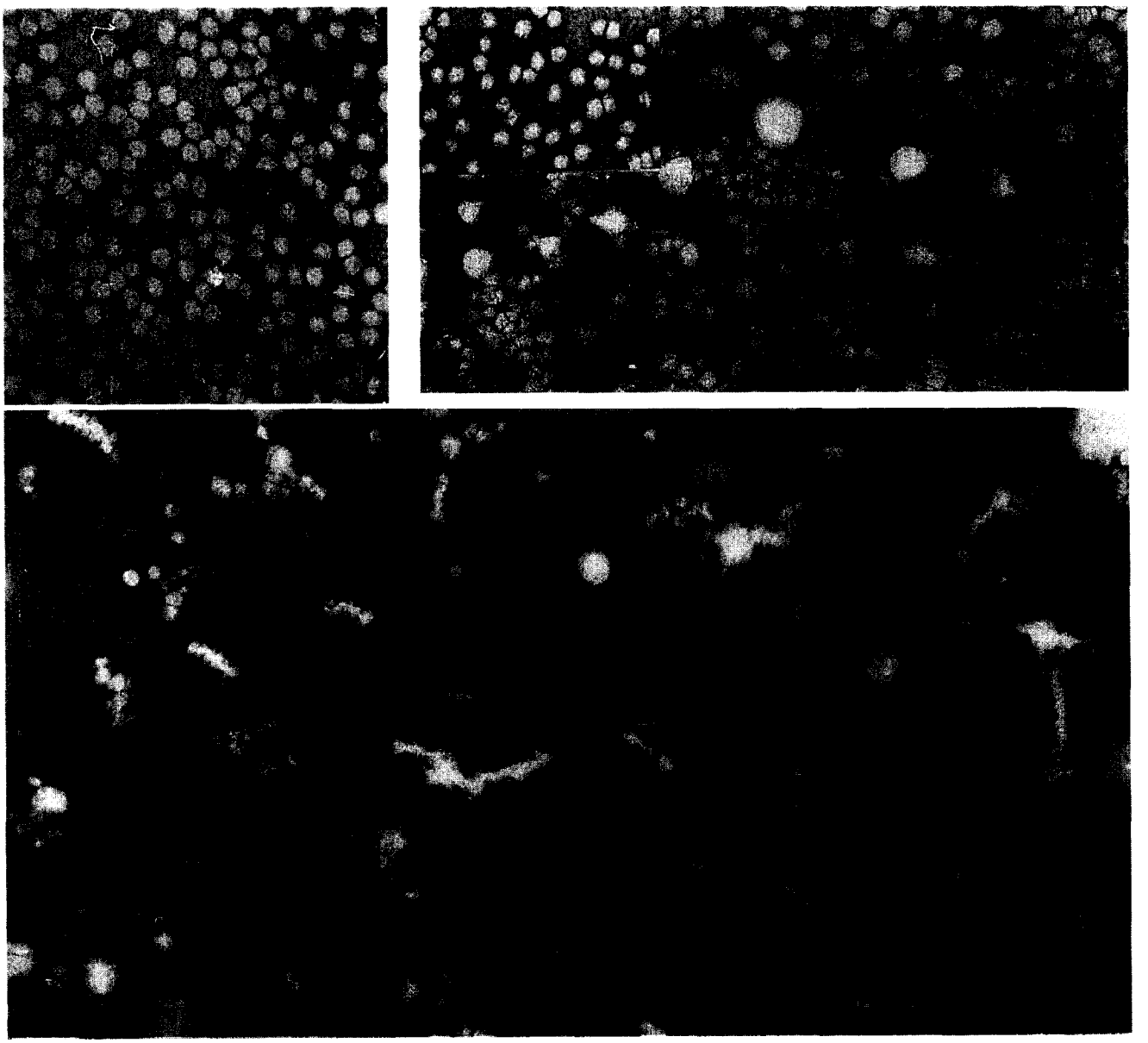

Fig. 1. Effect of AGE-Alb on the morphology of normal human serum LDL (nLDL) incubated in oxidative conditions. Negative staining electron microscopy showed that, as compared to LDL particles prior to the incubation (a), after exposure to 1 μM $CuSO_4$ for 24 h, at 37°C, the particles were slightly aggregated (arrow), some appearing as compact droplets (d) or as vesicles (arrowhead) (b). Native albumin added to the incubation medium prevents the changes in LDL morphology (b, inset). Addition of AGE-Alb to the incubation medium induced substantial changes in LDL that appeared as rouleaux (r), fused compact droplets (d) and aggregates (arrow). Few monomeric LDL particles (m) are also present (c). a–c: Bar = 100 nm.

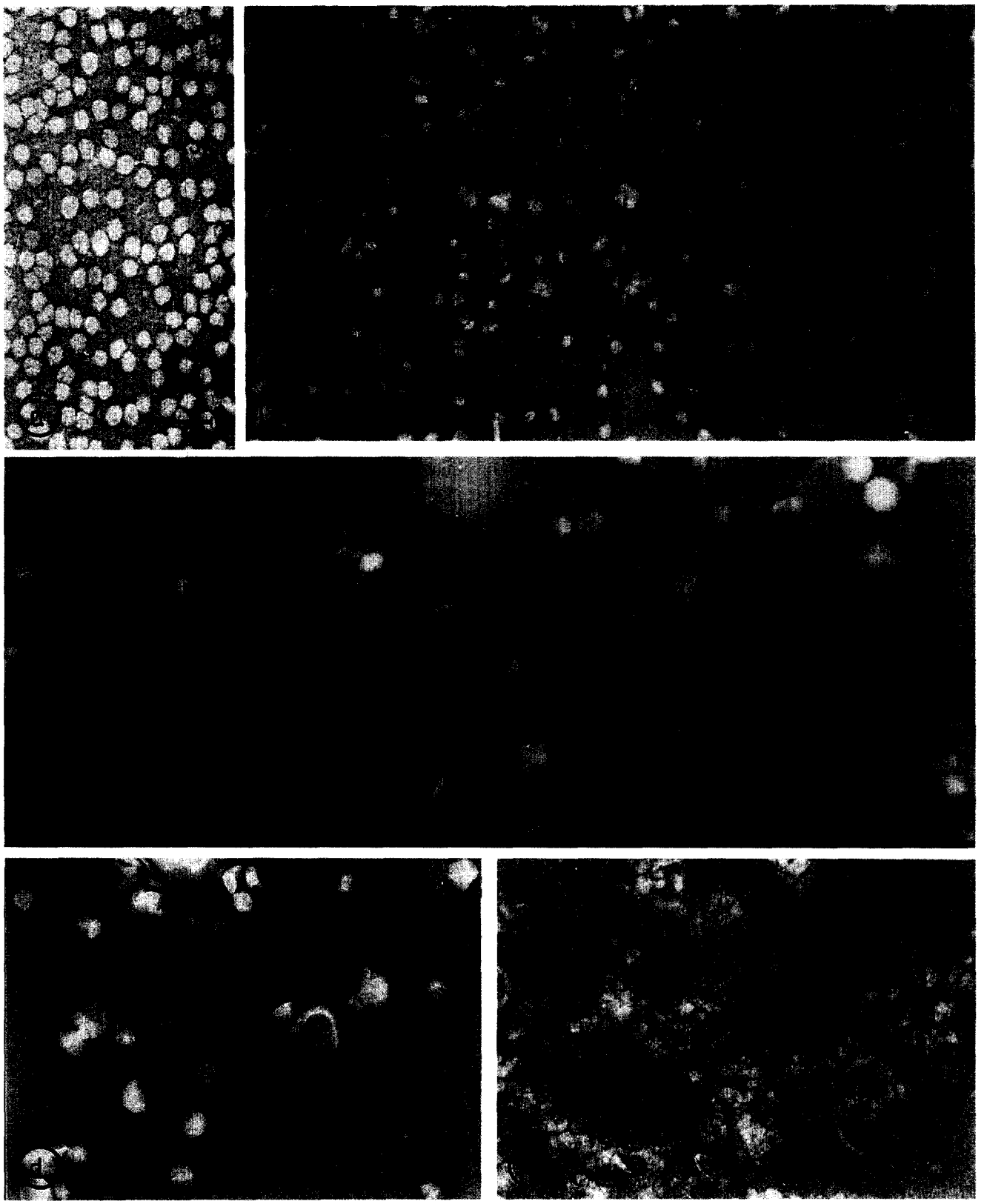

Fig. 2. Effect of AGE-Alb on LDL isolated from diabetic patients (dLDL) (protein ratio (w/w) LDL/AGE-Alb = 1). Negatively stained micrographs revealed that while the native probes consisted in monomers only (a), after 24 h incubation with 1 μM $CuSO_4$ at 37°C, dLDL appeared as compact droplets (d), vesicles (v) and monomers (m) (b). In the presence of AGE-Alb, drastic modifications and massive reorganization of the particles occur: dLDL appeared mostly as lipid droplets (d) and collapsed vesicles (v) (c and d) and sometimes as large aggregates (e). a–e: Bar = 100 nm.

6.8) containing 2% SDS and 5% β-mercaptoethanol. Aliquots (10–25 μg) were applied on 5–15% SDS-polyacrylamide gradient gel [29] and electrophoresed at a constant current of 10 mA for 14 h. Gels were stained with 0.1% Coomassie Brilliant Blue G-250 in 50% methanol and 10% acetic acid.

Lipid analysis

LDL cholesterol was measured chemically [30] or enzymatically using a Sigma kit. Unesterified and esterified cholesterol were quantitated after solvent extraction [31] and subsequent separation by thin-layer chromatography (TLC). The latter was performed on precoated plates using petroleum ether-diethylether-acetic acid (90:10:1) as developing system. Upon exposure to, and visualization with iodine vapours, the spots corresponding to the lipid standards (run on the same plates) were scraped from the gel [32] and chemically measured [30]. Solvent blanks and blank areas from each TLC plate were measured and subtracted from the values of corresponding probes. Phospholipid classes were quantitated as in [33] after their separation by TLC according to Skipski [34]. Lipid phosphorus was converted to phospholipid using a multiplication factor of 25. Appropriate blanks and controls were included for each lipid class assay.

Negative staining electron microscopy

Samples of LDL obtained after incubation in each of the above conditions were placed on grids and stained with 1% sodium phosphotungstate adjusted to pH 8.0 [35]. For each experimental condition, 3–5 grids of each sample were examined with a Philips 201C and a 400 HM electron microscope.

3. Results

3.1. Characterization of probes

Native LDL appeared on SDS-PAGE as a single protein band at the expected position of apo B. The level of glycation of nLDL (collected from 64 subjects) or dLDL (38 subjects) determined using hydroxymethyl furfural (HMF) as standard according to the method in [20] had indicated values of 160 ± 24 nmol HMF/mol apoB for nLDL and of 270 ± 50 nmol HMF/mol apoB for dLDL. In the case of NIDDM patients, the level of LDL glycation was constantly higher than that of normal subjects ($P < 0.001$ when compared with normal subjects).

3.2. AGE-Alb effect on LDL morphology

Samples of nLDL and dLDL, freshly isolated or after incubation with AGE-Alb in different oxidative conditions, and isolated again (to remove AGE-Alb) were examined by negative staining electron microscopy. As expected, native nLDL appeared as monomeric particles ranging in size between 20 and 23 nm (Fig. 1a). Upon incubation for 24–48 h at 37°C in either autoxidative conditions or with 1 μM copper, nLDL appeared as monomers, as aggregated or fused particles (Fig. 1b). When subjected to similar conditions but in the presence of AGE-Alb significant changes in LDL morphology occurred. In addition to rare monomers, nLDL often appeared as large aggregates, rouleaux and fused particles; these changes were particularly prominent in the case of Cu^{2+}-induced oxidation (Fig. 1c). In contrast to AGE-Alb, addition of Alb to the incubation mixture preserved the native appearance of LDL (Fig. 1, inset).

When subjected to conditions similar to those described above, dLDL was even more drastically modified by AGE-Alb (Fig. 2). Isolated dLDL particles were not different from nLDL in negative staining (Fig. 2a). However, upon oxidation, besides monomers, vesicles and fused particles were present (Fig. 2b). These modifications were abolished by addition of native albumin to the incubation medium. By contrast, addition of AGE-Alb during incubation induced even more severe alteration in the dLDL morphology that appeared mainly as vesicles, collapsed and fused particles (Fig. 2c,d) and occasionally as large aggregates, consisting possibly of partially delipidated apo B deposits and excess surface lipids (Fig. 2e); only few monomeric particles were still present (Fig. 2c,d).

3.3. AGE-Alb effect on the TBARS and conjugated dienes generation during nLDL or dLDL oxidation

nLDL

When freshly isolated nLDL was maintained for 24 h at 37°C, the level of lipid peroxides expressed as TBARS corresponded to a mild oxidation (~ 10 nmol MDA/mg

Table 1
Effect of AGE-Alb on TBARS and conjugated dienes generation during oxidation of normal LDL (nLDL)

Experimental conditions [a]	TBARS (nmol MDA/mg protein LDL)	Conjugated dienes ΔA (234 nm) [b]
nLDL: freshly isolated	0.8 ± 0.3	-
nLDL: autoxidation, 24 h	10.2 ± 3.1	0.81 ± 0.03
nLDL: autoxidation + AGE-Alb	4.0 ± 1.2	0.59 ± 0.07
nLDL: autoxidation + Alb	3.1 ± 1.1	0.62 ± 0.09
nLDL + Cu^{2+} (24 h)	33.0 ± 1.4	1.04 ± 0.04
nLDL + Cu^{2+} + Alb	4.3 ± 1.1	0.73 ± 0.05
nLDL + Cu^{2+} + AGE-Alb	6.1 ± 1.4	0.79 ± 0.05

[a] LDL (0.1 mg/ml) was subjected to autoxidation at 37°C for 24 h in the absence or presence of AGE-Alb or Alb. The lipoprotein: albumin weight ratio was 1:1. Values represent the average of three experiments.
TBARS, thiobarbituric acid reactive substances; MDA, malondialdehyde; Alb, bovine serum albumin; AGE-Alb, advanced glycosylated albumin.
[b] Conjugated dienes are expressed as the increase in absorbance at 234 nm of each probe. All probes had concentrations of 0.5 mg LDL protein/ml.

Table 2
Effect of AGE-Alb on generation of TBARS and conjugated dienes during oxidation of diabetic LDL (dLDL)

Experimental conditions [a]	TBARS (nmol MDA/mg protein LDL)	Conjugated dienes ΔA (234 nm) [b]
dLDL: freshly isolated	1.8 ± 0.4	-
dLDL: autoxidation, 24 h	26.4 ± 4.5	0.99 ± 0.16
dLDL: autoxidation + AGE-Alb	8.4 ± 1.4	0.73 ± 0.09
dLDL: autoxidation + Alb	4.3 ± 0.6	0.76 ± 0.03
dLDL + Cu^{2+} (24 h)	36.1 ± 1.7	1.44 ± 0.10
dLDL + Cu^{2+} + Alb	7.6 ± 2.2	0.83 ± 0.09
dLDL + Cu^{2+} + AGE-Alb	9.5 ± 3.5	0.95 ± 0.06

[a] The mixture containing dLDL (0.1 mg/ml) and without or in the presence AGE-Alb or Alb (0.1 mg/ml) was incubated with 1 μM $CuSO_4$, for 24 h at 37°C.
TBARS, thiobarbituric acid reactive substances; MDA, malondialdehyde; Alb, bovine serum albumin; AGE-Alb, advanced glycosylated albumin; dLDL, diabetic LDL.
[b] Conjugated dienes are expressed as in Table 1.

protein). The presence of Alb or AGE-Alb in the incubation mixture, reduced by 60% and 70%, respectively, the TBARS level (Table 1). Peroxidation of nLDL in the presence of copper gave a value of ~ 33 nmol MDA/mg protein, indicating an extensive lipid peroxidation. The process was efficiently reduced by addition of Alb or AGE-Alb in the incubation medium (Table 1).

dLDL

When freshly isolated, dLDL had constantly a higher level of TBARS than nLDL (~ 1.8 and ~ 0.8 nmol MDA/mg protein LDL, respectively). Subjected to the same conditions as above (autoxidation or Cu^{2+}-induced oxidation), the level of TBARS values detected for dLDL were always higher than those for nLDL. As in the case of nLDL, the generation of TBARS was significantly reduced when AGE-Alb or Alb were added to the incubation mixture (Table 2).

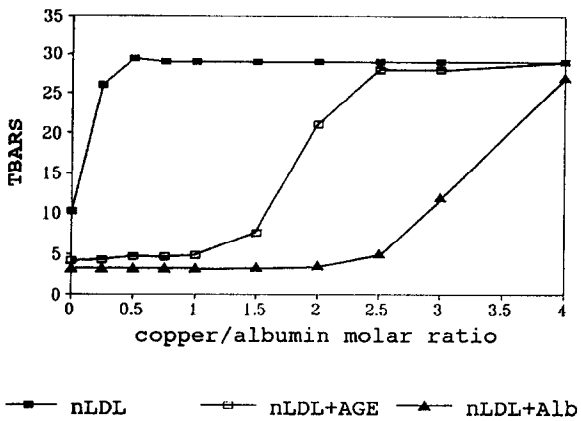

Fig. 3. Dependence on copper concentration of LDL peroxidation in the presence of AGE-Alb or Alb, expressed as TBARS generation. The albumins were dialyzed for 24 h at 4°C against Tris-buffered saline (pH 7.4), containing 0.1% EDTA (to remove possible impurification with copper), followed by extensive dialysis in Tris-buffered saline (pH 7.4) without EDTA. For all experiments, the albumin/protein LDL ratio was 1:1 (w/w), whereas the concentration of copper ions:albumin molar ratio was increased from 0 to 4. Note that AGE-Alb ceases to prevent LDL peroxidation at the molar ratio of 1, whereas albumin is effective up to the copper/albumin molar ratio of 2.5.

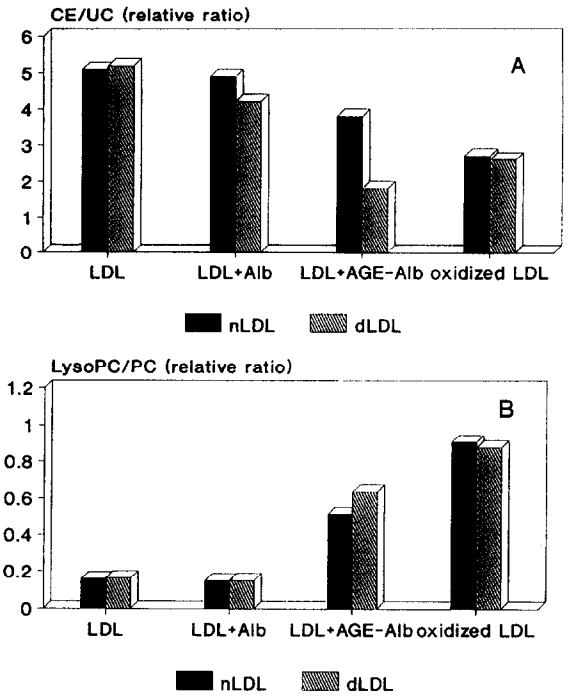

Fig. 4. Changes in the ratio between lipid classes of nLDL and dLDL incubated in oxidative conditions (1 μM copper sulfate) in the presence of AGE-Alb or Alb. (A) Variation in the CE/UC relative ratio determined by thin layer chromatography and colorimetric assay. (B) Modification of relative ratio between phospholipid classes. CE = cholesteryl esters; UC = unesterified cholesterol; PC = phosphatidylcholine; LysoPC = lysophosphatidylcholine. Values represent the average of four experiments.

In all experimental conditions described above, addition of oxidation inhibitors (1 mM EDTA and 0.22 μM BHT) maintained the TBARS value at the level of the native probes. Determination of conjugated dienes indicated similar variations as for TBARS (Tables 1 and 2).

These experiments and the emerging data indicated that at Alb/LDL protein weight ratio of 1:1, AGE-Alb and especially Alb prevent the generation of TBARS in the absence or presence of Cu^{2+}. To find out whether this effect was dependent on Cu^{2+} concentration, additional procedures were carried out using various Cu^{2+}/albumin molar ratio (from 0 to 4) while maintaining a constant 1:1 albumin/LDL protein weight ratio. These experiments showed that the antioxidant effect of native albumin was efficient up to a Cu^{2+}/albumin molar ratio of 2.5. This threshold was significantly lower for AGE-Alb that inhibited the generation of lipid peroxidation up to the molar ratio of copper/AGE-Alb of 1:1 (Fig. 3). As the molar ratio of copper ions to AGE-Alb went above 1, the TBARS attained gradually the same values as those of LDL oxidized for 24 h at 37°C (Fig. 3). This is possibly due to a

reduced capacity of AGE-Alb to bind Cu^{2+} ions, as already reported for glycated Alb [36].

3.4. AGE-Alb effect on LDL chemistry

Analysis of major lipid classes of nLDL and dLDL incubated in oxidative conditions (1 μm $CuSO_4$) revealed that, both free cholesterol and lysophosphatidylcholine content were greatly augmented as compared with the respective native lipoproteins. Consequently, the cholesterol ester/unesterified cholesterol (CE/UC) ratio was decreased, whereas the lysophosphatidylcholine/phosphatidylcholine (LysoPC/PC) ratio was increased (Fig. 4A and B). As expected, addition of native Alb to the incubation medium preserved these lipid ratios at normal values for both nLDL and dLDL (Fig. 4). By contrast, analysis of lipid content of nLDL and dLDL oxidized in the presence of AGE-Alb showed important alterations comparable to but different from those detected in oxidized lipoproteins in the absence of AGE-Alb. As compared to normal LDL, the CE/UC ratio decreased ~ 1.3-fold for nLDL and ~ 3-fold for dLDL. The LysoPC/PC ratio increased ~ 3-fold for nLDL and ~ 3.5-fold for dLDL (Fig. 4B). In all experiments these changes were more prominent in the case of dLDL. Similar results were obtained in experiments in which gAlb was removed from AGE-Alb preparation (by GlycoGel B chromatography) this suggested that, if present, gAlb had no influence on AGE-Alb-induced modifications of lipoprotein.

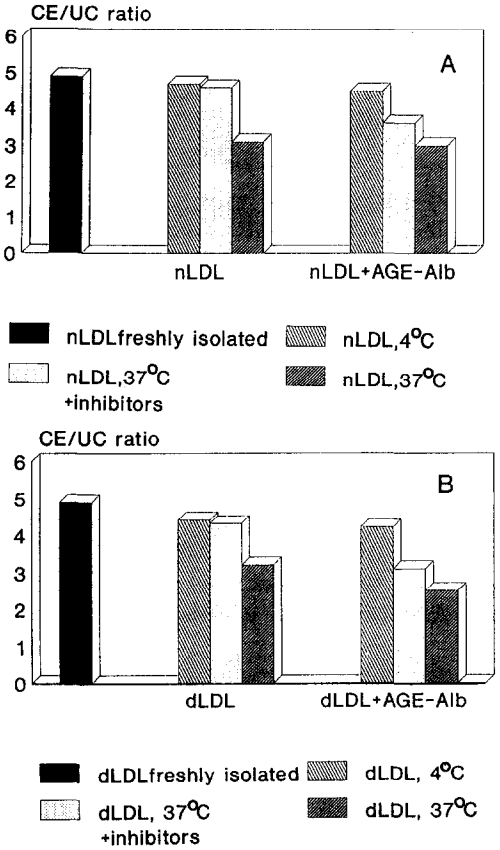

Fig. 5. CE/UC ratio of nLDL (A) and dLDL (B) incubated for 48 h in the absence or presence of AGE-Alb, in the following conditions: at 4°C, at 37°C and at 37°C in the presence of oxidation inhibitors (EDTA and BHT). Note that the presence of inhibitors does not prevent the AGE-Alb to affect the lipid composition of nLDL and especially dLDL. CE = cholesteryl esters; UC = unesterified cholesterol. The results are from one representative experiment.

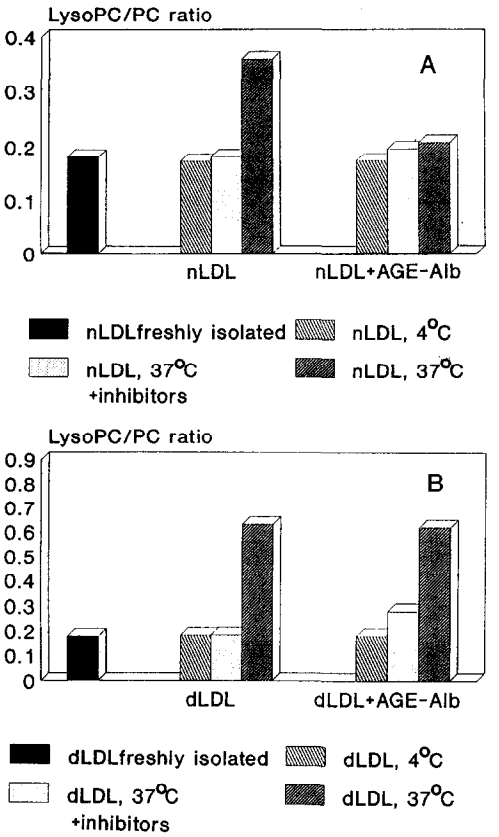

Fig. 6. LysoPC/PC ratio of nLDL (A) and dLDL (B) incubated in various conditions (4°C, 37°C, 37°C with oxidation inhibitors EDTA and BHT) for 48h, with or without AGE-Alb. The weight ratio AGE-Alb/LDL = 1:1. Note the slight increase (~ 1.5-fold) in the relative phospholipid ratio of dLDL exposed to AGE-Alb, even in the presence of oxidation inhibitors. LysoPC = lysophosphatidylcholine; PC = phosphatidylcholine. Results are from one representative experiment.

Taken together, these results showed that in oxidative conditions, AGE-Alb induced modifications in the lipid content of nLDL and dLDL which are not accompanied by a significant increase in TBARS level (Tables 1 and 2).

These surprising findings led us to address the issue whether AGE-Alb by itself may induce physico-chemical alterations of LDL particles, irrespective of the presence of oxidative conditions. To this purpose, we incubated either nLDL or dLDL with AGE-Alb at 37°C in the absence or presence of oxidation inhibitors (EDTA and BHT) or at 4°C. As previously mentioned, in the latter two conditions the TBARS values were similar to those of the native particles. Incubation of nLDL for 48 h either at 4°C or at 37°C in the presence of inhibitors, had no effect on CE/UC ratio. As expected, incubation at 37°C in the

absence of inhibitors (autoxidation) decreased by ~33% the CE/UC ratio (Fig. 5A). This is probable due to a mild oxidation of the lipoproteins, a result that is in good agreement with the increase in TBARS values detected in such conditions. Addition of AGE-Alb to nLDL samples in the three conditions tested (37°C, 37°C with inhibitors and at 4°C), showed that at 4°C there is no significant change in CE/UC ratio, as compared with nLDL; however, at 37°C a characteristic decrease of ~24% and ~37% was detected in the presence or absence of inhibitors, respectively (Fig. 5A). The situation was somehow similar for dLDL, but the CE/UC ratio was even lower (~40%) when AGE-Alb was added at 37°C in the presence of oxidation inhibitors (Fig. 5B).

Determination of LysoPC/PC ratio indicated that in the absence of AGE-Alb no change occurred at 4°C and 37°C in the presence of EDTA and BHT; however, in the absence of inhibitors at 37°C, a ~2-fold increase and a ~3-fold increase of this ratio was obtained for nLDL and dLDL, respectively (Fig. 6A, B). When AGE-Alb was added to nLDL, the LysoPC/PC ratio was not significantly modified (Fig. 6A). In the absence of inhibitors, the effect of AGE-Alb on dLDL was not different than that observed when dLDL was incubated without AGE-Alb. However, a slight (1.5-fold) increase in the LysoPC/PC ratio was recorded in the presence of inhibitors.

Since AGE-Alb induced significant alterations in the lipid composition of lipoproteins even in the presence of inhibitors of oxidation, we can assume that AGE-Alb by itself was responsible for these modifications. It was obvious that in all cases, dLDL was more susceptible and reactive than nLDL, probably because minimal, undetectable intrinsic modifications are present in dLDL molecule.

Protein analysis by SDS-PAGE revealed that apo B-100 was the only protein present in nLDL whereas some fine lower molecular weight protein bands were present in freshly isolated dLDL (Fig. 7a,b). When subjected to copper-induced oxidation, the electrophoretic pattern of both nLDL and dLDL showed drastically fragmented apo B; addition of native Alb prevented in part the degradation of apo B especially in the case of nLDL suggesting a mild breakdown of the protein. The degradative process was more pronounced for dLDL (Fig. 7a,b). In contradistinction, the incubation of the probes in the presence of AGE-Alb had a severe effect on the protein. On SDS-PAGE nLDL exhibit numerous lower molecular weight peptide fragments ranging between 120 and 250 kDa. In the case of dLDL, the degradation of apo B was more prominent and only few protein bands of an apparent molecular mass of 66 and 58 kDa were present (Fig. 7b).

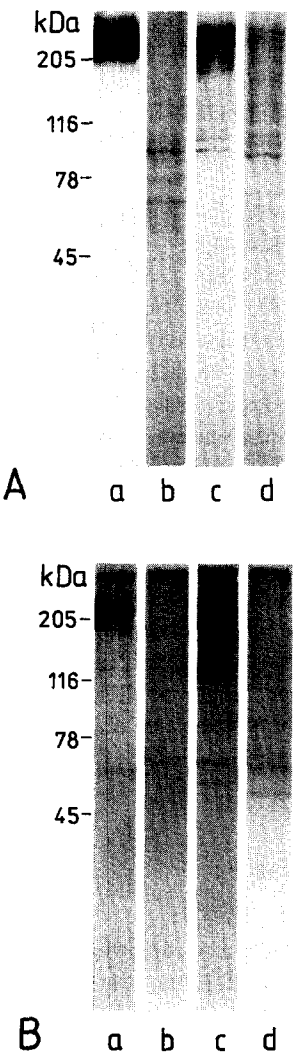

Fig. 7. SDS-PAGE of LDL obtained from normal (nLDL) or diabetic subjects (dLDL) oxidized in the presence of AGE-Alb or albumin (Alb) and further isolated by ultracentrifugation (to remove Alb or AGE-Alb). (A) Freshly isolated nLDL (a) oxidized for 24 h (b), in the presence of albumin (c) or AGE-Alb (d). (B) dLDL, before oxidation (a), after 24 h oxidation (b), in the presence of Alb (c) or of AGE-Alb (d). The latter significantly affects the apo B especially of dLDL.

4. Discussion

The experiments reported here, demonstrate that: (a) AGE-Alb directly induces structural and chemical modifications of human nLDL and dLDL; (b) these modifications are enhanced in oxidative conditions without significant changes in the TBARS level; (c) in all experimental conditions dLDL is more susceptible to the AGE-Alb-induced changes.

We choose to study the interaction between AGE-Alb and LDL in an in vitro system, so as to avoid any interference of other cellular or extracellular components. Initially, we tested whether AGE-Alb still has the antioxidant potential on LDL peroxidation as already reported for albumin [15]. The results indicated that, in terms of TBARS generation, the situation is to a certain extent similar to that of Alb, in both cases the levels detected being low. However, there is a clear difference with respect to the threshold of Cu^{2+}/albumin ratio, which for AGE-Alb is

2.5-times lower than for Alb. This finding corroborates well with published data indicating that glycated albumin has a diminished binding capacity for copper ions [5,36]. Interestingly, short term (14 days) reversibly glycated albumin (gAlb), upon incubation with nLDL and dLDL in the conditions described for AGE-Alb, generates a significant amount of TBARS, without notable change in the lipid composition of lipoproteins (unpublished observations). These data suggest possible different mechanisms of action of gAlb and AGE-Alb on LDL molecule.

It was intriguing that in the absence of significant oxidative modifications, at least in terms of generation of TBARS (or conjugated dienes), the morphology of LDL particles was drastically changed, the lipid composition modified and the apo B integrity altered. Thus, upon exposure to AGE-Alb, in oxidative conditions, nLDL and especially dLDL appear aggregated, vesiculated, or as collapsed fused particles. These modifications are accompanied by a significant increase in unesterified cholesterol (UC) and lysophosphatidyl choline (LysoPC) and a drastic degradation of apo B. The strong effect of AGE-Alb on dLDL, in particular in oxidative conditions (Fig. 4A), may be due to the lipoprotein higher concentration in TBARS, and to the increased glycation of apo B (as compared to normal LDL) that intensify dLDL susceptibility to these aggressive factors. These changes could be due either to (i) interference of AGE-Alb in TBARS determination (the detailed chemistry of AGE products is rather unknown) and in fact, oxidation still occur, or (ii) that the modifications observed are due to the direct effect of the AGE-Alb on LDL particles without generation of augmented amounts of TBARS. To eliminate the potential influence of oxidation, we designed a series of experiments in which copper ions were omitted and nLDL or dLDL were incubated with AGE-Alb either at 37°C in the presence of oxidation inhibitors, or at 4°C. These experiments showed that AGE-Alb, even in the presence of EDTA and BHT, produced morphological and chemical alterations of LDL molecule. Consequently, it is safe to conclude that AGE-Alb by itself alters the normal or diabetic LDL, and the oxidative conditions enhance these modifications.

There were no changes in lipoprotein structure when experiments were performed at 4°C. These suggest the possible involvement of an enzymatic-like process which occur at 37°C only; it was reported that in certain conditions apo B exhibits a phospholipase A2-like activity [37]. The pattern of protein fragmentation, constantly observed in AGE-Alb-exposed LDL, could be, at least in part, the result of a phospholipase-like activity of apo B. It was also demonstrated that, in certain conditions, albumin exerts an esterase-like activity [38]. We consider the possibility that conformational changes induced by advanced glycosylation in the Alb molecule could intensify albumin's esterase-like activity, and consequently could explain a raise in the UC content of LDL. This possibility was tested by incubating Alb or AGE-Alb with reconstituted [^{14}C]cholesteryl oleate LDL and assessing the ^{14}C-free fatty acids released [39]; no differences between Alb and AGE-Alb were detected.

In general, the AGE-Alb induced similar modifications of LDL either in the absence or in the presence of oxidative conditions, but the latter enhances clearly these alterations. Furthermore, in all experiments, the structural and chemical changes were stronger for dLDL suggesting that the molecule is more labile and predisposed to further alterations. It has been reported that LDL from NIDDM patients is significantly more glycosylated as compared to that of normal subjects [40] and that in elderly subjects hyperinsulinemia could be correlated with the presence in plasma of a more consistent fraction of small, dense, atherogenic LDL [41]. Nevertheless, as indicated by Lyons et al., in diabetic patients the increase in the glycative stress is not always associated with an inherent increase in the oxidative stress [40]. Still, accelerated atherosclerosis is frequently associated with diabetes. Our results support these data, and suggest that extended glycation, rather than oxidation of lipoproteins, at least at the onset of the process, makes LDL prone to AGE-Alb induced major modifications.

It has been shown that both LDL and AGE-Alb transcytose the endothelial layer [42,43]. One can assume that within the arterial wall of diabetic subjects the transcytosed dLDL and AGE-Alb may interact and rapidly generate modified forms of lipoproteins similar to those we have detected as the result of their in vitro interaction. Similar forms of modified and reassembled lipoproteins were observed in the arterial intima of hypercholesterolemic animals [44,45] and are believed to be highly atherogenic, inducing the foam cell formation. We assume that the rapid and drastic changes of glycated lipoproteins that occur in the presence of AGE-Alb could result in the formation of modified lipoproteins in the arterial wall of diabetic subjects (in the absence of hypercholesterolemic diet or strong prooxidant conditions), which could be one of the events that trigger accelerated atherosclerosis, the most common complication of diabetes.

Acknowledgements

We gratefully acknowledge the helpful discussion with Dr. David Stern (Department of Physiology and Cellular Biophysics, Columbia University, New York, USA), and the excellent technical assistance of E. Florea (biochemistry), M. Daju (word processing), E. Stefan (photography) and C. Neacsu (graphics). We also thank M. Serpe for help in some biochemical assays. This work was supported by the Romanian Academy and the USA-NIH Fogarty International Center Grant-TW00118, UNESCO contract SC/RP 201066.

References

[1] Brownlee, M., Cerami, A. and Vlassara, H. (1988) New Engl. J. Med. 318, 1315–1321.
[2] Vlassara, H., Brownlee, M. and Cerami, A. (1986) Clin. Chem. 32, B37–B41.
[3] Yagi, K. (1987) Chem. Phys. Lipids 45, 337–351.
[4] Wolff, S.P. and Dean, R.T. (1987) Biochem. J. 245, 243–250.
[5] Shaklai, N., Garlick, R.L. and Bunn, M.F. (1989) J. Biol. Chem. 259, 3812–3817.
[6] Okamoto, G., Hayase, F. and Kato, H. (1992) Biosci. Biotech. Biochem. 56, 928–931.
[7] Sasky, J. and Cottam, G.L. (1982) Biochim. Biophys. Acta 713, 199–207.
[8] Baynes, J.W. (1991) Diabetes 40, 405–412.
[9] Lyons, T.J., Patrick, J.S., Baynes, J.W., Colwell, J.A. and Lopes-Virella, MF. (1986) Diabetologia 29, 685–689.
[10] Yla-Herttuala, S., Palinski, W., Rosenfeld, M.E., Parthasarathy, S., Carew, T.E., Butler, S., Witztum, J.L. and Steinberg, D. (1989) J. Clin. Invest. 84, 1086–1095.
[11] Morel, D.W., Hessler, J.R. and Chisolm, G.M. (1983) J. Lipid Res. 24, 1070–1076.
[12] Steinbrecher, U.P., Parthasarathy, S., Leake, D.S., Witztum, J.L. and Steinberg, D. (1984) Proc. Natl. Acad. Sci. USA 81, 3883–3887.
[13] Halliwell, B. (1988) Biochem. Pharmacol. 37, 569–571.
[14] Halliwell, B. and Gutteridge, J.M.L. (1989) Free Radicals in Biology and Medicine, pp. 254–288, Clavendon Press, Oxford.
[15] Dobrian, A., Mora, R., Simionescu M. and Simionescu, N. (1993) Biochem. Biophys. Acta 1169, 12–24.
[16] Naito, C., Nakamura, M. and Yamamoto, Y. (1993) Ann. N.Y. Acad. Sci. 676, 27–45.
[17] Iberg, N. and Fluckiger, R. (1986) J. Biol. Chem. 261, 13542-13545.
[18] Mateo, M.C.M., Bustamante, J.B. and Cantalapiedra, M.A.G. (1978) J. Biomed. 29, 56–58.
[19] Redgrave, T.G., Roberts, D.C.K. and West C.E. (1975) Anal. Biochem. 65, 42–49.
[20] Murtiashaw, M.H., Young, J.E., Strickland, A.L., McFarland, K.F., Thorpe, S.R. and Baynes, J.W. (1983) Clin. Chim. Acta 130, 177–187.
[21] Monnier, V.M. and Cerami, A. (1981) Science 211, 491–493.
[22] Vlassara, H., Brownlee, M. and Cerami, A. (1985) Proc. Natl. Acad. Sci. USA 82, 5588–5592.
[23] Yatscoff, R.W., Mehta, A., Gerrard, J.M. and Thliveris, J. (1987) Clin. Biochem. 20, 359–363.
[24] Schuh, J., Fairclough, G.F. Jr. and Haschemeyer, R.H. (1978) Proc. Natl. Acad. Sci. USA 75, 3173–3177.
[25] Esterbauer, H., Jurgens, G., Queheberger, O. and Koller, E. (1987) J. Lipid Res. 28, 495–509.
[26] Fogelman, A.M., Schechter, I., Seager, J., Hokom, M., Child, J.S. and Edwards, P.A. (1980) Proc. Natl. Acad. Sci. USA 77, 2214–2218.
[27] Esterbauer, H., Striegl, G., Fuhl, H. and Rotheneder, M. (1989) Rad. Res. Commun. 6, 67–75.
[28] Markwell, M.A.K., Hass, S.M., Beihr, I.E. and Tolbert, N.E. (1978) Anal. Biochem. 87, 205–209.
[29] Maizel, V.J. (1971) Methods Virol. 5, 179–246.
[30] Zak, B. (1957) Am. J. Clin. Pathol. 27, 583–587.
[31] Bligh, E.G. and Dyer, V.J. (1959) Can. J. Biochem. Physiol. 37, 911–917.
[32] Ames, B.N. and Dubin, D.T. (1960) J. Biol. Chem. 235, 769–775.
[33] Skipski, V.P., Peterson, R.F. and Barclay, M. (1964) Biochem. J. 90, 374–378.
[34] Skipski, V.P. and Manning, J.A. (1968) Enzymology 1, 530–598.
[35] Forte, T.M. and Nordhausen, R.W. (1971) Methods Enzymol. 128, pp. 442–457.
[36] Di Simplicio, P., Cheeseman, K.H. and Slater, T.F. (1991) Free Rad. Res. Commun. 14, 253–262.
[37] Leake, D.S., Witztum, J.L. and Steinberg, P. (1984) Proc. Natl. Acad. Sci. USA 81, 3883–3887.
[38] Kurano, Y., Kushida, I., Tanaka, H. and Ikeda, K. (1992) Chem. Pharm. Bull. 40, 2169–2172.
[39] Krieger, M., McPhane, M.J., Goldstein, J.L. and Brown, M.S. (1979) J. Biol. Chem. 254, 3845–3863.
[40] Lyons, T.J. (1993) Am. J. Cardiol. 71, 26B–31B.
[41] Mykkanen, L., Kuusisto, J., Haffner, S.M., Pyorala, K. and Laakso, M. (1994) Arterioscler. Thromb. 14, 518–526.
[42] Vasile, E., Simionescu, M. and Simionescu, N. (1983) J. Cell. Biol. 96, 1677–1689.
[43] Schmidt, A.M., Hasu, M., Popov, D., Zhang, J.H., Chen, J., Yan, S.D., Brett, J., Cao, R., Kuwabara, K., Costache, G., Simionescu, N., Simionescu, M., Stern, D. (1994) Proc. Natl. Acad. Sci. USA 1994 (in press).
[44] Simionescu, N., Vasile, E., Lupu, F., Popescu, G. and Simionescu, M. (1986) Am. J. Pathol. 123, 109–125.
[45] Mora, R., Simionescu, M. and Simionescu, N. (1990) J. Lipid Res. 31, 1793–1807.